

Article

Modulation of Antimicrobial Peptide–Membrane Interactions by Lysyl-Phosphatidylglycerol in *Staphylococcus aureus*: An FTIR Spectroscopy Study

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Abstract

Changes in membrane lipid composition constitute a key bacterial resistance mechanism. In *Staphylococcus aureus*, phosphatidylglycerol undergoes lysine modification to form lysyl-phosphatidylglycerol, a cationic lipid that reduces the net negative surface charge and thereby enhances resistance to cationic antimicrobial peptides. In this study, we examined the influence of lysyl-PG on the membrane activity of three antimicrobial peptides with distinct physicochemical characteristics: LL-37, F5W Magainin II, and NA-CATH:ATRA-1-ATRA-1. Model membranes composed of phosphatidylglycerol and cardiolipin were supplemented with increasing molar fractions of lysyl-phosphatidylglycerol, and peptide–membrane interactions were characterized using Fourier-transform infrared spectroscopy. Membrane fluidity was evaluated through shifts in the symmetric methylene stretching bands, while changes in interfacial polarity were assessed via the carbonyl and phosphate asymmetric stretching bands. LL-37 induced pronounced disruption of anionic bilayers, an effect progressively attenuated by lysyl-phosphatidylglycerol, particularly within the hydrophobic core. F5W Magainin perturbed both hydrophobic and interfacial regions across a broader range of lysyl-phosphatidylglycerol concentrations, whereas NA-CATH:ATRA-1-ATRA-1 primarily targeted interfacial domains, with minimal disruption of acyl chain order. Increasing lysyl-PG content modulated the extent of bilayer disorder and dehydration at the hydrophobic–hydrophilic interface, with each peptide exhibiting a distinct interaction profile. Collectively, these findings provide mechanistic insights into lysyl-PG-mediated modulation of peptide activity and highlight the role of lipid remodeling as a bacterial defense strategy.

Keywords: *Staphylococcus aureus*; antimicrobial peptides; lysyl-PG; peptide–membrane interaction; infrared spectroscopy; membrane models



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1. Introduction

The membrane lipid composition of *Staphylococcus aureus* (*S. aureus*) is highly dynamic and central to its ability to persist in hostile environments, including those encountered during infection [1]. Lipid composition is tightly regulated to balance membrane stability, fluidity, and charge [2–7]. The major lipid classes of *S. aureus* are phosphatidylglycerol (PG), an anionic lipid that provides the baseline negative surface charge of the bacterial membrane [8]; cardiolipin (CL), an enriched dimeric phospholipid that has been suggested to play a critical role in stabilizing protein complexes, adapting to stress conditions, and

regulating cell division [9,10]; and lysyl-phosphatidylglycerol (lysyl-PG). The last of these is considered a key adaptive mechanism of the bacteria. Positively charged lysyl-PG is produced by the modification of anionic phosphatidylglycerol with L-lysine, mediated by the multiple peptide resistance factor (MprF) enzyme. The synthesis of lysyl-PG modulates the negative membrane surface charge and has been associated with a resistance mechanism against cationic antimicrobial peptides (AMPs) [11–13].

AMPs are typically small, amphipathic molecules which are part of the innate immune response in several organisms [14]. They are considered a promising alternative to conventional antibiotics as their mechanism of action is primarily driven by electrostatic interactions with negatively charged surfaces, such as bacterial membranes [15,16]. This charge-based interaction confers selectivity, allowing AMPs to preferentially bind to bacterial membranes over mammalian membranes [14]. Once bound to the membrane, AMPs can disrupt its integrity through multiple mechanisms, including the formation of transmembrane pores or by accumulating on the surface and disrupting the bilayer in a detergent-like manner, as proposed in the carpet model [17]. However, it has been extensively reported that several bacteria, including *S. aureus*, modify their lipid composition as part of resistance mechanism against exogenous agents. The most classical and widely supported explanation is that resistance arises from electrostatic repulsion. *S. aureus* modulates the charge of the cytoplasmic membrane, resulting in a decrease in the negative charge of the bacterial surface.

Although lysyl-PG has been included in some previous biophysical studies, its role in peptide–membrane interactions remains insufficiently characterized, particularly at the molecular level. In this study, the effect of increasing concentrations of lysyl-PG in a representative membrane model of *S. aureus* and the impact of these on the interaction with the peptides LL-37, NA-CATH: ATRA-1-ATRA-1 (NA), and F5W Magainin II (F5W Mag) was explored. These peptides were selected based on their physicochemical characteristics and biological activity against *S. aureus*. With the goal of investigating the effects of increased lysyl-PG content on the hydrophobic core and polar interface of the membrane, Fourier-transformed infrared spectroscopy (FTIR) was used. This approach enables a detailed assessment of how membrane lipid composition governs peptide–lipid interactions, with implications for understanding the resistance mechanisms.

2. Materials and Methods

2.1. Reagents

1,2-dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DMPG, Lot. 140PG-164), 1',3'-bis [1,2-dimyristoleoyl-sn-glycero-3-phospho-glycerol sodium salt (CL, Lot. 750332P-200MG-A-030), and 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-3-lysyl 1-glycerol chloride salt (Lysyl PG. Lot 840520P-5MG-D-011) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). HPLC-grade methanol and chloroform were purchased from Merck (Kenilworth, NJ, USA). HEPES was purchased from Sigma-Aldrich (St. Louis, MO, USA), NaCl from Carlo Erba (Val de Reuil, NOR, FR), and EDTA from Amresco (Solon, OH, USA).

Peptides LL-37 (Lot. V1440EE070/PE1324), F5W Magainin II (Lot U2431HE110-3/PE4465), and NA-CATH: ATRA-1-ATRA-1 (Lot U037QFC180-11/PE6100) were synthesized according to their reported sequences using the solid-phase method, and purchased from GenScript (Piscataway Township, NJ, USA). The purity of the peptides was determined by analytical HPLC ($\geq 95\%$). Their molecular weight was confirmed by MALDI-TOF mass spectrometry.

2.2. Prediction of the 3D Structure of Peptides Under Study

Using the PEP-FOLD3 web server [18], the three-dimensional structures of the peptides LL-37, F5W Mag, and NA were obtained from their primary sequences (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>, accessed on 20 May 2025). PEP-FOLD3 integrates a Hidden Markov Model-structured alphabet (SA-HMM) and a coarse-grained force field tailored for short peptides to predict structures. This approach is especially effective for peptides that have the potential to gain defined secondary structures, such as α -helices, when interacting with membranes, as they are easier to predict [19]. PEP-FOLD3 provided ten candidate models for each peptide. The model chosen for further analysis had the lowest energy score which, in this case, meant that it was the most stable under physiological conditions. The online tool NetWheels was used to model the helical wheel diagrams of the peptides studied (<http://lbqp.unb.br/NetWheels>, accessed on 23 May 2025). To do this, the sequence of each peptide was input into the application, and a diagram was generated [20].

2.3. Phase Transition Measurements by Infrared Spectroscopy

Supported lipid bilayers (SLBs) were prepared in situ using a BioATR II cell connected to a Tensor II spectrometer (Bruker Optics, Ettlingen, Germany), equipped with a liquid nitrogen-cooled MCT detector. Spectra were collected at a resolution of 0.4 cm^{-1} , averaging 120 scans per spectrum for each sample. Temperature control was provided by a Huber Ministat 125 water bath (Huber, Offenburg, Germany), offering $\pm 0.01\text{ }^{\circ}\text{C}$ precision. Background spectra were recorded under identical conditions using HEPES buffer (20 mM HEPES, 500 mM NaCl, and 1 mM EDTA). Silicon crystals were coated by applying $20\text{ }\mu\text{L}$ of lipid stock solutions prepared in chloroform, whose composition depended on the membrane models used as follows: DMPG:CL (80:20 *w/w*) or DMPG:CL:Lysyl-PG mixtures at 72:18:10, 64:16:20, or 56:14:30 *w/w* ratios. After loading the cells with the lipid solution, the chloroform was evaporated to form a multilayer lipid film. For in situ analysis, $20\text{ }\mu\text{L}$ of either buffer or peptide solution was added, followed by a 10 min incubation at a temperature above the lipid phase transition. To evaluate the vibrational shifts, the absorbance spectra were truncated to the $2970\text{--}2820\text{ cm}^{-1}$ range (for CH_2 bands), the $1760\text{--}1700\text{ cm}^{-1}$ range (for $\text{C}=\text{O}$ bands), and the $1265\text{--}1140\text{ cm}^{-1}$ range (for PO_2^- bands). The data were baseline-corrected and analyzed using the peak-picking tool in the OPUS 8.8.4 software. Peak positions were plotted against temperature, and the lipid phase main transition temperature values were derived by fitting the thermal transition curves to the Boltzmann model. The main transition temperature values were identified at the inflection points of each curve using OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA). An additional procedure was performed with the PO_2^- bands, which were deconvoluted into 12 peaks in the OPUS software. The maximum peak position was selected considering the ranges described in the literature for each lipid in each phase. Then, with the values plotted, smoothing was performed using the Fast Fourier Transform (FFT) filter, which reduced noise and obtained sigmoidal curves using OriginPro.

3. Results

3.1. Prediction of the 3D Structure of the Peptides

The physicochemical parameters of the peptides LL-37, F5W Mag and NA are summarized in Table 1. LL-37, known as human cathelicidin, is the only cathelicidin synthesized in humans [21]. In aqueous solutions, LL-37 has no secondary structure. However, it adopts an amphipathic α -helical conformation in the presence of physiological salt concentrations or upon interacting with membranes [22–24]. This structural feature allows LL-37 to insert into membranes and, depending on the peptide-to-lipid ratio, adopt mechanisms

such as pore formation [25]. The antimicrobial peptide F5W Mag is a synthetic analog of Magainin II, a cationic α -helical peptide naturally found in the skin of the African clawed frog (*Xenopus laevis*) [26]. Finally, NA is a modified peptide derived from the peptide NA-CATH, which in turn is isolated from the species *Naja atra* or Chinese cobra [27–29].

Table 1. Physicochemical and biological properties of antimicrobial peptides.

Peptide	Sequence	Charge	Hydrophobicity (%)	MIC (μ M)	EC ₅₀ (μ M)	Ref
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	+6	37.8	5.99	0.25	[21,29]
F5W Mag	GIGKWLHSAKKFGKAFVGEIMNS	+3	43.5	6.49	-	[30]
NA	KRFKKFFKKLKNVKKRFFKKFFKKLKVIGVTFPF	+15	44.1	-	0.09	[29]

Data obtained from Peptide 2.0 (https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php, accessed on 20 May 2025) for hydrophobicity/hydrophilicity analysis and PepCalc (<https://pepcalc.com/>, accessed on 20 May 2025) for charge.

To better understand the structural features that may influence membrane interactions, the helical wheel and secondary structure predictions of LL-37, F5W Mag, and NA were analyzed. According to the helical wheel projections (Figure 1a–c) all three peptides adopted amphipathic conformations, with hydrophobic and cationic polar residues distributed on opposite faces, which is a typical arrangement for membrane-active antimicrobial peptides. This distribution pattern could influence the association of each peptide with lipid bilayers, particularly in terms of electrostatic interaction to negatively charged bacterial membranes and the potential for insertion into the hydrophobic core.

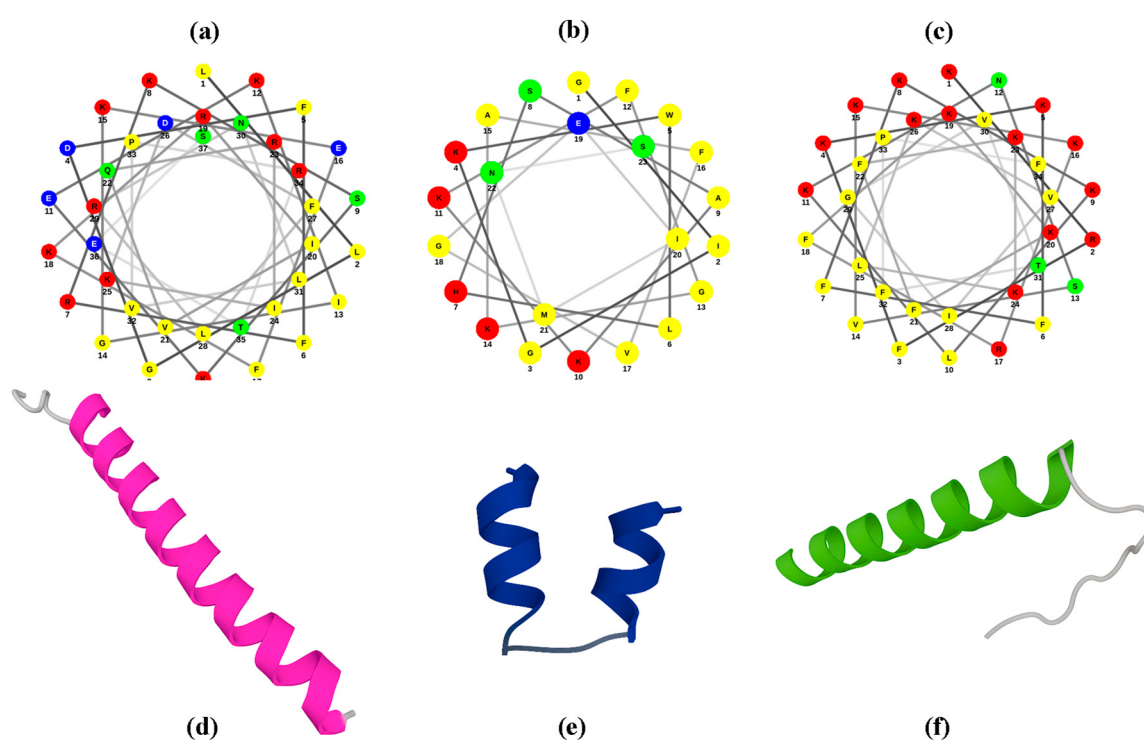


Figure 1. Helical wheel diagrams for (a) LL-37, (b) F5W Mag, and (c) NA. Polar residues are represented as follows: basic—red; acidic—blue; and uncharged—green. Nonpolar residues are represented in yellow. Prediction of three-dimensional structures of (d) LL-37, (e) F5W Mag, and (f) NA.

According to the structural predictions obtained from the PEPFOLD3 online platform, all three peptides adopted amphipathic α -helical conformations, with hydrophobic and

cationic polar residues distributed on opposite faces, which is a typical arrangement for membrane-active antimicrobial peptides. The predicted 3D models (Figure 1d–f) indicate that LL-37 and NA adopt a unique α -helices, whereas F5WMag forms two α -helical segments separated by a loop.

3.2. Phase Transition Experiments by Infrared Spectroscopy

AMPs' mechanism of action is intrinsically associated with their capacity to perturb lipid bilayer structure and dynamics through interactions with both the hydrophobic core and polar headgroup region of membranes. To examine these effects, FTIR spectroscopy was employed, since it provides a powerful approach for monitoring lipid phase behavior and structural transitions in model membrane systems [31]. The technique makes it possible to monitor the vibrational signatures corresponding to distinct bilayer regions: acyl chains (hydrophobic core), ester carbonyls (interfacial region), and phosphate groups (headgroup region) [32–34]. The symmetric stretching vibration of the methylene groups ($\nu_s\text{CH}_2$) is a well-established spectroscopic marker for assessing the conformational order of lipid acyl chains within bilayers. Monitoring the temperature dependence of this band allows the detection of the lipid phase transition [35]. At low temperatures the gel phase (L_β) lies at 2850 cm^{-1} and at higher temperature the liquid crystalline phase (L_α) is located close to 2853 cm^{-1} . Shifts in this band to higher wavenumbers indicate an increase in the gauche conformations relative to the trans conformations, which reflects the enhanced chain disorder and fluidization of the membrane. The temperature at which the phase transition occurs is called the main transition temperature (T_m).

Since AMPs initially interact with the surface of the phospholipid bilayer, their influence on the interfacial region and headgroup was also analyzed [36,37]. The vibrational modes corresponding to the C=O and PO_2^- groups serve as sensitive reporters of molecular changes at the interface [38]. In this context, shifts in these bands reflect alterations in hydrogen bonding, hydration state, and headgroup conformation, which are typically induced by molecular adsorption, insertion, or electrostatic interactions with the bilayer surface [39,40]. At the polar/apolar interface, changes were tracked through the ester carbonyl stretching vibration ($\nu\text{C=O}$), typically observed between 1760 and 1700 cm^{-1} [37,41]. This group acts as a bridge between the phosphate head groups and the acyl chains of phospholipids. A shift to lower wavenumbers generally indicates increased hydrogen bonding or an increase in the polarity of the environment, whereas a shift to higher wavenumbers suggests dehydration or perturbation of the interfacial region by bound peptides [34,42–44]. Finally, the asymmetric stretching vibration of the phosphate group ($\nu_{\text{as}}\text{PO}_2^-$), located at approximately 1265 – 1141 cm^{-1} , is a marker for studying the lipid–water interface and molecular interactions in biological membranes. A shift to higher wavenumbers in the $\nu_{\text{as}}\text{PO}_2^-$ band often indicates dehydration or a decrease in hydrogen bond formation of the phosphate group with water [45,46]. This may be due to competition for binding sites by other molecules or lipid packing, which excludes water [47,48]. These changes can be interpreted as evidence of peptide binding to the membrane surface, which may alter peptide–lipid interactions, induce headgroup rearrangements, or disrupt hydration shells [49–52].

The first step was to obtain the phase transition of the pure DMPG, CL, and Lysyl-PG, the results of which are presented in the Supplementary Data (Figure S1). The following step was to obtain the phase transitions of the multicomponent lipid systems and their interaction with LL-37, F5WMag, and NA. The results of the interaction of the three peptides with DMPG:CL (80:20) systems are presented in Figure 2. The symmetric methylene stretching and the carbonyl stretching band revealed clear peptide-dependent changes in acyl chain order.

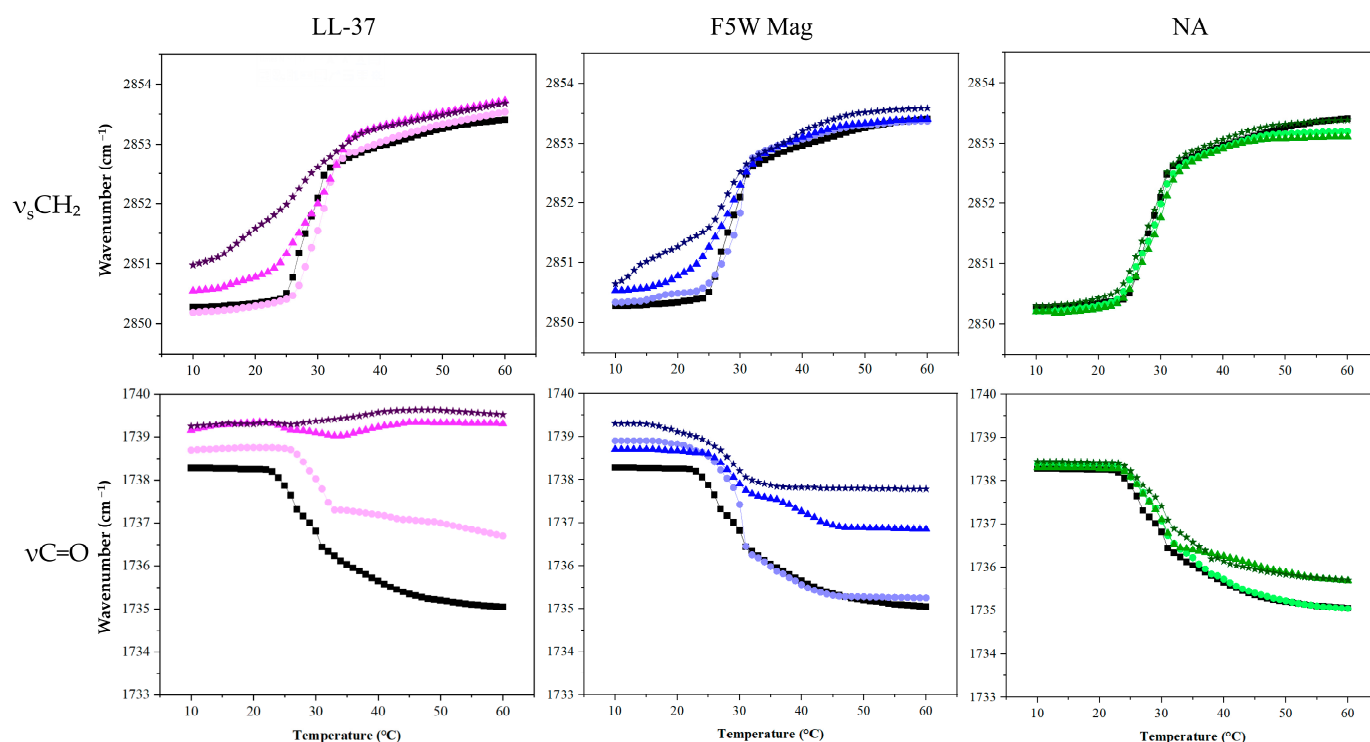


Figure 2. Peak positions of the symmetric methylene stretching ($\nu_s\text{CH}_2$, **top** panels) and carbonyl stretching ($\nu\text{C}=\text{O}$, **bottom** panels) bands of DMPG:CL (80:20) membranes in the absence of peptide (■) and presence of increasing concentrations (1%, 5%, 10%) of LL-37 (pink), F5W-magainin (blue), and NA (green). Symbols correspond to different peptide concentrations: 1% (●), 5% (▲), and 10% (★). All spectra were recorded in buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, and pH 7.4).

In the binary system ($T_m = 28.76 \pm 0.15$ °C), LL-37 exhibited the strongest membrane-disruptive activity. Increasing peptide concentrations induced a marked upshift of the $\nu_s\text{CH}_2$ vibration, indicating acyl chain disorder and reduced cooperativity during the gel-to-liquid crystalline transition. This was consistent with a decrease in T_m of up to 26.02 °C at 10 mol% LL-37 (Table 2). F5W also perturbed the hydrophobic core, producing concentration-dependent increases in $\nu_s\text{CH}_2$, particularly in the gel phase. At 10 mol%, F5W lowered the transition temperature to 27.77 ± 0.33 °C. The behavior is similar to but less pronounced than that of LL-37. In contrast, NA had minimal effects on the acyl chains, with no significant changes in $\nu_s\text{CH}_2$ or in T_m across the concentrations tested (Table 2). At the interfacial level, LL-37 induced strong upward shifts in $\nu\text{C}=\text{O}$ and had the strongest effect, suggesting dehydration of the hydrophobic/hydrophilic zone and displacement of water molecules at the lipid interface. F5W also produced concentration-dependent upward shifts in $\nu\text{C}=\text{O}$ that were more pronounced in the crystalline phase, indicating early peptide insertion and progressive interfacial dehydration. In comparison, NA induced only mild increases in carbonyl wavenumbers at 5 and 10 mol%, consistent with limited interfacial dehydration and peptide localization near the polar/apolar region. This effect was accompanied by shifts in the phosphate band, shown in the Supplementary Material (Figure S2), that suggest electrostatic interactions with the headgroups and further loss of interfacial water for LL-37. Also, with F5W Mag, corresponding changes in the phosphate region reinforced the trend of $\nu\text{C}=\text{O}$. Nonetheless, NA promoted concentration-dependent changes in the phosphate bands, particularly in the crystalline phase, confirming preferential interaction at the headgroup level.

Table 2. Phase transition temperatures (T_m) of the different lipid systems in the presence of increasing concentrations of the peptides, as determined by FTIR. The standard deviation was ± 0.01 °C.

Peptide	Concentration (mol%)	T_m (°C)			
		DMPG:CL (80:20)	(72:18:10)	DMPG:CL:Lysyl-PG (64:16:20)	(56:14:30)
LL-37	0	28.76 $\pm$ 0.15	35.65 $\pm$ 0.14	38.57 $\pm$ 0.13	43.20 $\pm$ 0.21
	1	30.32 $\pm$ 0.23	34.99 $\pm$ 0.24	38.06 $\pm$ 0.28	43.12 $\pm$ 0.17
	5	29.82 $\pm$ 0.31	36.40 $\pm$ 0.30	37.33 $\pm$ 0.37	41.71 $\pm$ 0.12
	10	26.02 $\pm$ 0.55	36.52 $\pm$ 0.20	41.12 $\pm$ 0.34	40.64 $\pm$ 0.12
F5W Mag	0	28.76 $\pm$ 0.15	35.65 $\pm$ 0.14	38.57 $\pm$ 0.13	43.20 $\pm$ 0.21
	1	29.53 $\pm$ 0.13	32.99 $\pm$ 0.12	40.01 $\pm$ 0.20	37.56 $\pm$ 0.11
	5	28.27 $\pm$ 0.11	33.36 $\pm$ 0.12	36.32 $\pm$ 0.11	34.12 $\pm$ 0.20
	10	27.77 $\pm$ 0.33	35.43 $\pm$ 0.10	38.73 $\pm$ 0.07	44.24 $\pm$ 0.17
NA	0	28.76 $\pm$ 0.15	35.65 $\pm$ 0.14	38.57 $\pm$ 0.13	43.20 $\pm$ 0.21
	1	28.84 $\pm$ 0.11	34.86 $\pm$ 0.09	39.47 $\pm$ 0.09	43.12 $\pm$ 0.17
	5	29.32 $\pm$ 0.09	35.27 $\pm$ 0.08	40.79 $\pm$ 0.11	41.71 $\pm$ 0.12
	10	28.48 $\pm$ 0.12	34.55 $\pm$ 0.07	41.20 $\pm$ 0.10	40.64 $\pm$ 0.12

In the ternary system containing 10% lysyl-PG (Figure 3), the effect of the peptides on acyl chain order was modulated compared to the binary membranes. LL-37 produced only minor shifts in the $\nu_s\text{CH}_2$ band, with T_m values remaining stable across all concentrations, indicating that lysyl-PG neutralizes most of its fluidizing effect. In contrast, F5W induced a clear fluidization of the bilayer, with a concentration-dependent increase in $\nu_s\text{CH}_2$ and a decrease of up to 2.6 °C in T_m , consistent with reduced cooperativity during the phase transition. NA showed a weaker effect, producing only slight fluidization at higher concentrations, though still more pronounced than in the binary system. At the interfacial level, the carbonyl bands exhibited peptide-specific behaviors. LL-37 induced modest upward shifts in $\nu\text{C=O}$, suggesting persistent but weakened dehydration of the interface. F5W produced more substantial shifts, especially in the crystalline phase, reflecting stronger dehydration and accumulation of peptide at the polar/apolar interface. NA showed a stronger effect in this region in comparison to DMPG:CL system, with clear concentration-dependent increases in $\nu\text{C=O}$ wavenumbers at 1 and 10 mol%, consistent with enhanced localization and interfacial dehydration promoted by lysyl-PG.

Phosphate stretching vibrations (Figure S3) revealed a similar trend. LL-37 induced only minor shifts compared to the binary system, consistent with reduced electrostatic interactions in the presence of lysyl-PG. F5W continued to perturb this region, though with lower amplitude than in the binary membranes. NA produced marked concentration-dependent changes, especially at low temperatures, confirming a preferential interaction with headgroups and displacement of interfacial water.

In membranes containing 20% lysyl-PG (Figure 4), the peptides showed distinct effects on acyl chain order. LL-37 produced only mild fluidization at 1–5 mol%, reflected in a small decrease in T_m (up to 1.24 °C), while at 10 mol% it induced a slight rigidifying effect, increasing T_m relative to the control (Table 2). In contrast, F5W caused a strong concentration-dependent fluidization, lowering T_m from 38.57 ± 0.13 in the control to 36.32 ± 0.11 at 5 mol%. Once again, NA produced minimal changes in $\nu_s\text{CH}_2$, consistent with limited penetration into the hydrophobic core. At the interfacial level, all three peptides perturbed the carbonyl region, though with different intensities. LL-37 induced moderate upward shifts in $\nu\text{C=O}$, consistent with partial dehydration, while F5W caused pronounced shifts in both phases, reflecting strong dehydration and interfacial peptide accumulation. NA showed concentration-dependent changes, with clear increases in $\nu\text{C=O}$ wavenumbers

at higher peptide levels, confirming localization near the polar/apolar interface despite the absence of acyl chain disruption.

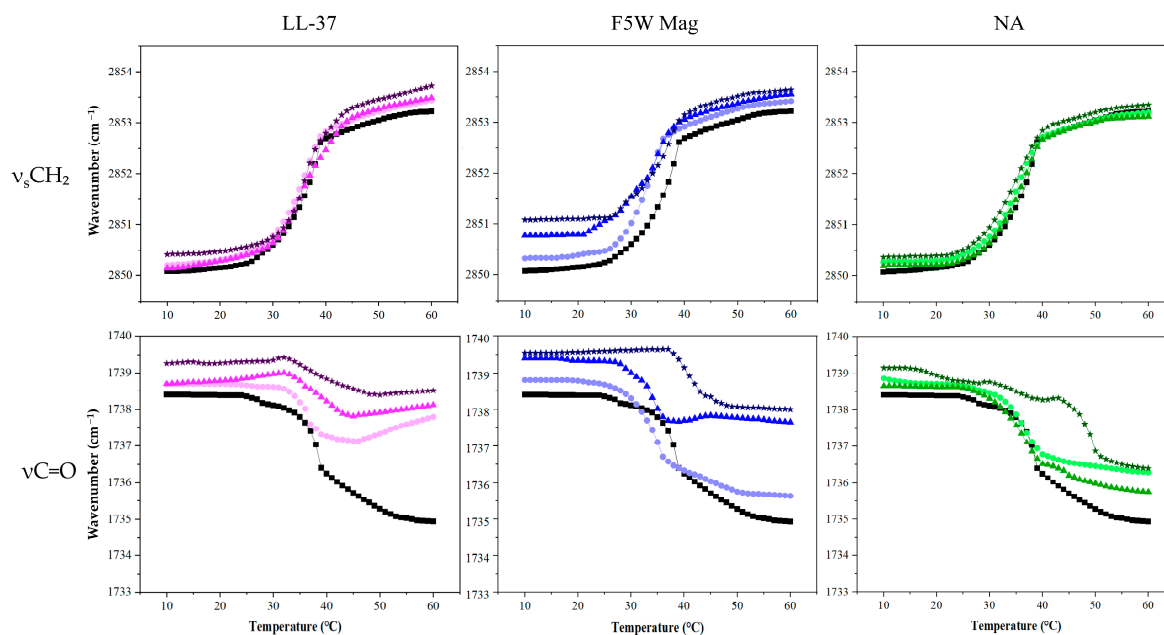


Figure 3. Peak positions of the symmetric methylene stretching ($\nu_s\text{CH}_2$, top panels) and carbonyl stretching ($\nu\text{C=O}$, bottom panels) bands of DMPG:CL:Lysyl-PG (72:18:10) membranes in the absence of peptide (■) and presence of increasing concentrations (1%, 5%, 10%) of LL-37 (pink), F5W-magainin (blue), and NA (green). Symbols correspond to different peptide concentrations: 1% (●), 5% (▲), and 10% (★). All spectra were recorded in buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, and pH 7.4).

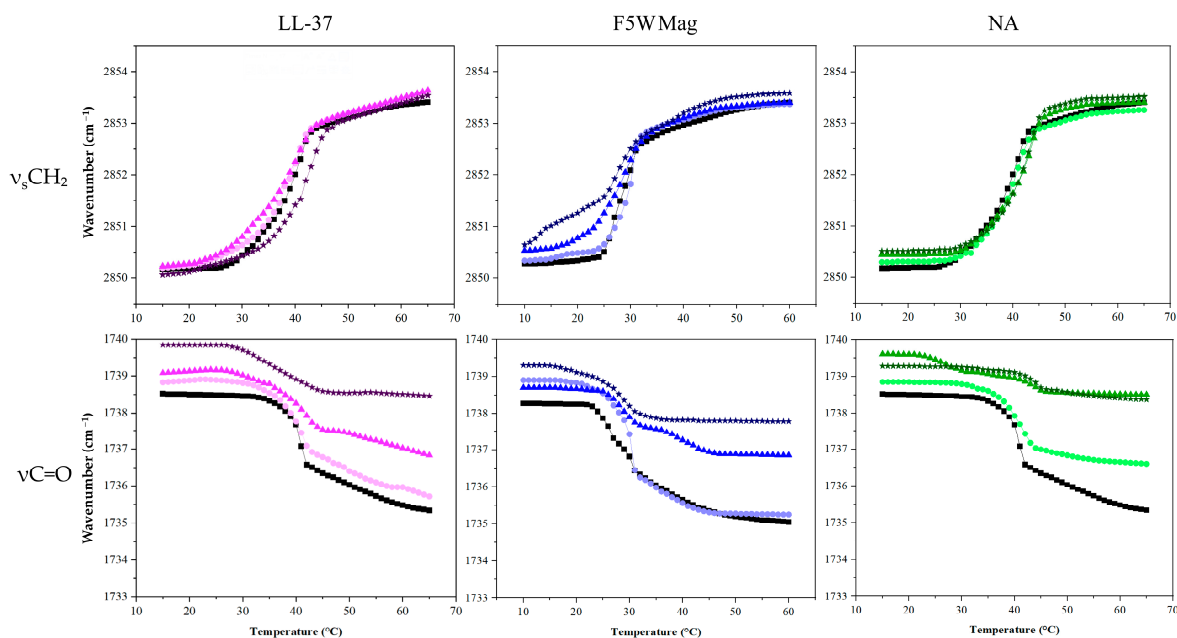


Figure 4. Peak positions of the symmetric methylene stretching ($\nu_s\text{CH}_2$, top panels) and carbonyl stretching ($\nu\text{C=O}$, bottom panels) bands of DMPG:CL:Lysyl-PG (64:16:20) membranes in the absence of peptide (■) and presence of increasing concentrations (1%, 5%, 10%) of LL-37 (pink), F5W-magainin (blue), and NA (green). Symbols correspond to different peptide concentrations: 1% (●), 5% (▲), and 10% (★). All spectra were recorded in buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, and pH 7.4).

Phosphate stretching vibrations (Figure S4) supported these trends. LL-37 produced smaller changes than in the 10% lysyl-PG system, suggesting attenuated electrostatic interactions. F5W continued to strongly perturb this region, whereas NA produced consistent shifts across concentrations, particularly in the crystalline phase, again highlighting its preferential interaction with interfacial headgroups.

At the highest lysyl-PG content of 30% (Figure 5), LL-37 produced only mild fluidization, with small decreases in T_m at all concentrations. When comparing this with lower lysyl-PG levels, it is evident that 30% lysyl-PG strongly attenuates its ability to disrupt acyl chain order. F5W, in contrast, retained the activity, inducing marked fluidization as evidenced by significant $\nu_s\text{CH}_2$ shifts, similar to those found with the 20% system. NA also perturbed the acyl chain region, lowering T_m from 43.20 ± 0.21 °C to 40.64 ± 0.12 °C at 10 mol%. In the interfacial region, LL-37 induced progressive $\nu\text{C=O}$ shifts to higher wavenumbers with increasing concentration, indicating persistent dehydration and strong interaction with headgroups in the crystalline phase. Similarly, F5W magainin caused interfacial perturbations, with both carbonyl and phosphate bands shifting to higher wavenumbers, confirming peptide accumulation at the bilayer surface. NA displayed a biphasic response in the carbonyl region-decreased wavenumbers at 1–5 mol% and increased values at 10 mol%-reflecting concentration-dependent dynamics at the polar/apolar interface. Phosphate vibrations reinforced these trends. LL-37 produced concentration-dependent $\nu_{\text{as}}\text{PO}_2^-$ shifts to higher wavenumbers (Figure S5), consistent with water displacement at the interface. F5W showed an effect similar to that observed at 20% lysyl-PG, while NA produced perturbations weaker than those found with 20% lysyl-PG, though still detectable in the crystalline phase. Overall, NA primarily perturbed the interfacial domains, with effects on carbonyl groups enhanced by moderate lysyl-PG content (10–30%).

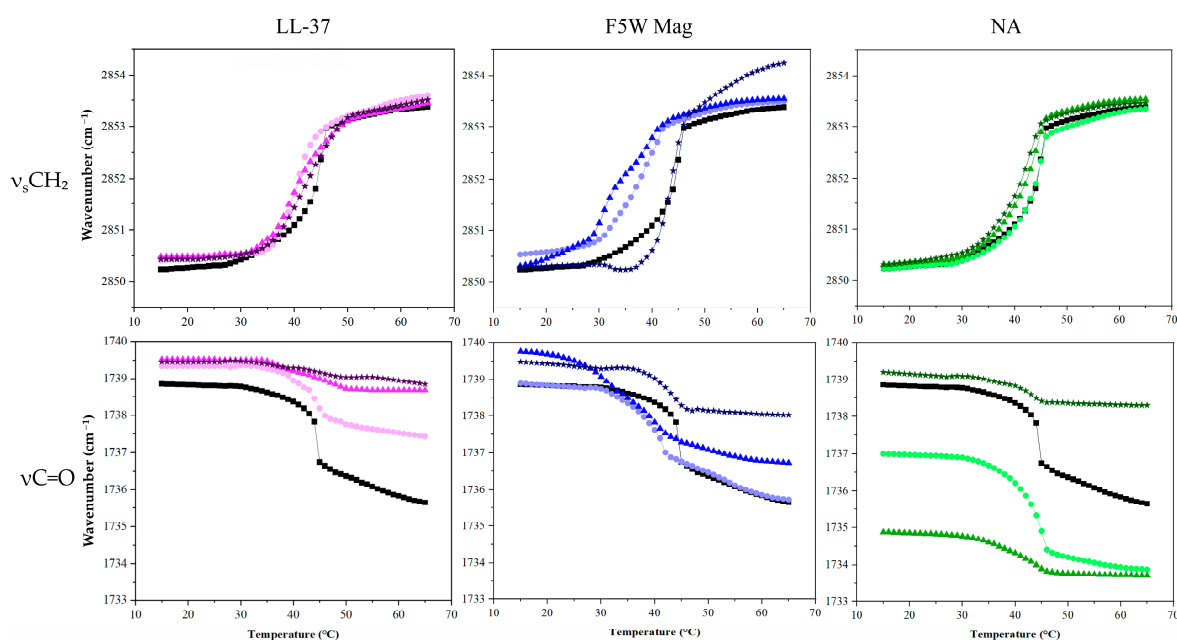


Figure 5. Peak positions of the symmetric methylene stretching ($\nu_s\text{CH}_2$, top panels) and carbonyl stretching ($\nu\text{C=O}$, bottom panels) bands of DMPG:CL:Lysyl-PG (56:14:30) membranes in the absence of peptide (■) and presence of increasing concentrations (1%, 5%, 10%) of LL-37 (pink), F5W-magainin (blue), and NA (green). Symbols correspond to different peptide concentrations: 1% (●), 5% (▲), and 10% (★). All spectra were recorded in buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, and pH 7.4).

Across all lipid systems, the three peptides displayed distinct interaction profiles that were progressively modulated by lysyl-PG content. LL-37 showed strong membrane-

disruptive activity in the binary system, fluidizing the acyl chains and dehydrating interfacial regions, but its effects were gradually attenuated as lysyl-PG increased, with only mild perturbations at 30%. F5W maintained broad activity across all systems, consistently inducing strong fluidization of the hydrophobic core and dehydration of interfacial groups, with only minor modulation by lysyl-PG. In contrast, NA exhibited minimal effects on the acyl chains in the binary system but increasingly perturbed interfacial regions (carbonyl and phosphate groups) as lysyl-PG levels rose, particularly at 10–30%. These results indicate that, while LL-37 is highly sensitive to lysyl-PG, F5W remains broadly active regardless of composition, and NA preferentially targets interfacial domains whose accessibility is enhanced by moderate lysyl-PG enrichment.

4. Discussion

Bacterial resistance to antimicrobial peptides is closely linked to adaptive modifications in membrane lipid composition. Such remodeling strategies alter the physicochemical properties of the bilayer, including surface charge, packing density, and fluidity, thereby reducing the efficacy of host defense peptides and therapeutic antimicrobials [7]. One of the best-characterized mechanisms in *S. aureus* involves the *mprF* gene, which mediates the lysinylation of PG to generate lysyl-PG. It has been proposed that this cationic lipid decreases the net negative charge of the membrane surface, leading to electrostatic repulsion of cationic antimicrobial peptides [13]. Previous studies have shown that disruption of *mprF* sensitizes *S. aureus* Sa113 to a broad range of host defense peptides, including defensins, protegrins 3 and 5, tachyplesin 1, and several AMPs such as magainin II and melittin [13]. Furthermore, *mprF* inactivation resulted in hypersusceptibility to daptomycin and decreased cytochrome C binding in *S. aureus* strains compared to the parental strain [53], supporting the charge repulsion mechanism.

Additionally, studies utilizing model membranes have revealed that this mechanism is more complicated. In certain instances, lysyl-PG exerts a negligible effect on peptide binding but markedly reduces membrane permeabilization. Furthermore, it was found that elevated lysyl-PG content condensed the bilayer, decreased PG availability, and increased the energy cost of peptide insertion [11,30]. Our FTIR results confirm that lysyl-PG modulates the interactions of LL-37, F5W Mag, and NA with bacterial membrane mimetics in a concentration-dependent manner, with distinct effects observed in the hydrophobic core, interface, and headgroup regions.

The predicted structure of LL-37 showed a α -helix with a slightly less ordered C-terminal region, consistent with previous reports, and a theoretical length of approximately 40 Å, which matches the average thickness of a lipid bilayer [54]. Previous studies widely reported that LL-37 binds to phospholipids and causes damage by pore forming [55,56]. The electrostatic attraction between the positively charged amino acids and negatively charged lipid headgroups is considered a critical first step, followed by hydrophobic interactions that drive deeper insertions. This dual interaction explains the capacity of the peptide to simultaneously perturb the hydrophobic acyl chains and polar interfacial regions, which destabilize lipid packing [25]. Previous circular dichroism (CD) analyses revealed that F5W Mag adopts an α -helical secondary structure in *S. aureus* mimetic vesicles containing 28% of lysyl-PG [30], and in PG and phosphatidylcholine vesicles at a 1:1 ratio a 63.9% helical structure was found, confirming that part of the peptide presents a random structure [57]. The prediction of the F5W Mag by PEP-FOLD3 reveals 2 helix connected by a loop; this flexible region has also been observed in other analogs of magainin II, such as Magainin-H₂ [58]. This flexibility may facilitate conformational adaptation during membrane interaction. In contrast, no structural studies are available for NA itself. However, data on the related cathelicidin NA-CATH indicate that it exhibits

helical character under membrane-mimetic conditions, such as in sodium dodecyl sulfate micelles, as revealed by CD [28]. Based on its sequence, net charge, and amphipathicity, NA is also expected to adopt an α -helical conformation upon interacting with lipid bilayers.

The FTIR results obtained in this study align with the established mechanism of LL-37. The strong acyl chain disorder induced by the incubation of the peptide and the dehydration effect observed in the interfacial groups in the fully anionic system reflect the interaction and the extensive disruption effect, which is consistent with earlier FTIR, NMR, and calorimetric studies reporting LL-37-induced lipid fluidization and headgroup dehydration [59–61]. The reduction in the membrane perturbation effect with increasing lysyl-PG content is also consistent with the expected effect of the changes in membrane lipid composition [7]. The results suggest that the presence of lysyl-PG causes LL-37 to remain superficially bound, primarily interacting with carbonyl groups rather than inserting to disrupt the hydrocarbon acyl chains. This modulation of the interaction may reflect a transition from a pore-forming or deeply penetrating mechanism in fully anionic membranes to a carpet-like surface perturbation in model membranes enriched with lysyl-PG. This interpretation aligns with previous studies showing that LL-37 preferentially localizes at the polar/apolar interface and disrupts hydration shells of water [62–64]. Both modes of action have been described for LL-37 in different lipid systems [24,65], which could contribute to its ability to act on a wide range of microbial targets while revealing its vulnerability to membrane modifications that attenuate electrostatic attraction. The presence of lysyl-PG appears to protect the membrane from LL-37, thereby limiting its insertion or its membrane-disruptive activity. This outcome supports the hypothesis that lysyl-PG impairs deep peptide insertion through charge neutralization and surface crowding, as observed in bacterial resistance mechanisms against cationic peptides [66,67].

F5W Mag exhibits slightly lower activity than LL-37 in *S. aureus*, as evidenced by the MIC values (Table 1); this is evident when comparing the results in the fully anionic system. Also, F5W Mag retains the broad-spectrum activity of Magainin II and exhibits enhanced hydrophobicity due to the substitution of phenylalanine with tryptophan at position 5. This modification allows tryptophan residues to be buried in the hydrophobic region of the bilayer, indicating a higher affinity for the membrane [68]. In bacterial membranes, such as those of *Bacillus megaterium*, F5W Mag forms well-defined toroidal pores of approximately 2.8–6.6 nm diameter [69]. FTIR results showed that F5W Mag perturbs both the hydrophobic and interfacial regions of the anionic system in a concentration-dependent manner. The peptide strongly fluidized the bilayer and displaced water molecules at the interface, consistent with the pore-forming mechanism described for F5W Mag in the literature [57,69,70].

Additionally, regarding physicochemical properties, F5W Mag is less charged and more hydrophobic than LL-37; this higher percentage of hydrophobicity in F5W is important in the interaction with systems containing lysyl-PG. It is observed that, unlike LL-37, lysyl-PG does not attenuate the interaction in the same way. The interaction and insertion of the antimicrobial peptide F5W Mag into *S. aureus* model membranes were strongly modulated by the lysyl-PG content. Rehal and coworkers demonstrated by neutron diffraction that F5W Mag penetrated both the headgroup and hydrophobic region of the bilayer in model membranes, mimicking the composition of *S. aureus* at pH 7.4 (with approximately 30% lysyl-PG). This insertion of the peptide caused a significant decrease in the bilayer d-spacing (4.6 Å) and a marked reduction in the Bragg intensity, indicating increased disorder and bilayer thinning [71]. In FTIR results, F5W Mag exhibited its highest membrane-disruptive activity at intermediate lysyl-PG levels (10–20%), where it induced pronounced disordering of the acyl chains and significant perturbations in the carbonyl and phosphate vibrational modes, indicative of both core and interfacial destabilization of

the bilayer. At higher lysyl-PG content (30%), the dehydration and interfacial disruption induced by the peptide were attenuated, indicating that lysyl-PG progressively shields the membrane from electrostatic engagement and destabilization.

These findings align with the attenuation mechanisms previously described under acidic conditions (high lysyl-PG content), where the formation of neutral ion pairs or anionic ion triplets with PG headgroups limits AMP insertion by stabilizing and tightening the membrane. Between these two lipids with opposite charges, the electrostatic interactions and Van Der Waals forces generate lipid complexes with a global charge, ion pairs with a globally neutral charge, or ionic triplets, in the last of which a single lysyl-PG molecule is positioned between two PG molecules, resulting in a complex with a net charge of -1 [72]. The formation of ion pairs promotes the ordering of the acyl chains, and stabilizes the condensed phase, although steric constraints in the equimolar mixed 1:1 system can result in slightly looser packing geometries and exhibit a significant steric effect on the packing geometry, which restricts molecular packing and reduces headgroup hydration [73,74].

In contrast, the formation of ion triplets, typically observed in lipid mixtures with anionic-to-cationic ratios close to 2:1 (PG/cationic surfactants or PG/P3adLPG/PG systems), results in notably more efficient chain packing than that of ion pairs or pure lipids. The delocalization of negative charge across two PG molecules relaxes the steric constraints present in ion pairs, resulting in a compact arrangement. Ion triplets strongly stabilized the gel phase, suppressed isothermal transitions, and increased the lipid melting temperature [72,73,75]. Consequently, membranes containing these complexes display enhanced resistance to AMP insertion, as the compact packing and reduced hydration of the interfacial region impose a physical barrier.

Finally, cathelicidin NA is a synthetic version of NA-CATH with two repeats of the ATRA-1 motif, which carries a net charge of $+15$ at physiological pH [28]. This high cationicity, combined with its 34-residue length and hydrophobicity of 44.1%, suggest that NA may insert into the hydrophobic core of lipid bilayers, as reported for other α -helical peptides of similar size such as maculatin [76]. Although hydrophobicities above 50% are often considered necessary for stable core insertion [77], NA approaches this threshold and may still achieve significant penetration because of its amphipathic α -helical structure.

However, our FTIR results indicate that NA interacts with membranes in a distinct manner to LL-37 and F5W Mag. Perturbation of the hydrophobic core was minimal, with only slight shifts in the methylene stretching bands and modest decreases in T_m values, even at the highest peptide concentration. In contrast, the most pronounced effects were observed in the interfacial region. The $\nu_{C=O}$ bands exhibited biphasic, concentration-dependent changes, with downshifts at 1–5 mol% and upshifts at 10 mol%, indicating dynamic interactions with carbonyl groups at the polar/apolar boundary. $\nu_{as}PO_2^-$ were less perturbed than in LL-37 or F5W Mag but still showed concentration-dependent upshifts in the crystalline phase, consistent with dehydration and peptide accumulation at the bilayer surface.

The presence of lysyl-PG further modulated these interactions. At moderate levels (10–20%), lysyl-PG enhanced interfacial perturbation, likely by reducing electrostatic repulsion and facilitating superficial peptide binding. At the highest lysyl-PG content (30%), NA induced only mild fluidization in the acyl chain region, while the carbonyls exhibited increased hydration at low concentrations. This behavior suggests that NA may adopt an orientation parallel to the bilayer surface, promoting interfacial disruption without deeply inserting into the hydrocarbon chains.

Of the evaluated peptides, NA displayed the highest activity, consistent with its elevated net positive charge ($+15$) and hydrophobicity, which favored strong interactions at the membrane interface, particularly with carbonyl groups at 30% lysyl-PG. NA also

showed greater potency than LL-37 in terms of EC_{50} [29], reinforcing the correlation between peptide activity and net charge, as highly cationic peptides interact more strongly with membrane regions, particularly with the carbonyl groups. LL-37, with an intermediate charge (+6) and lower hydrophobicity, exhibited only slightly higher activity than F5W Mag according to the MIC. However, F5W Mag, which is more hydrophobic than LL-37, displayed a higher capacity to perturb acyl chains and phosphate groups. These findings indicate that hydrophobicity is a key determinant of chain perturbation, while electrostatic charge primarily enhances interfacial engagement.

Taken together, our results highlight three distinct modes of action: LL-37 acts as a classical membrane active peptide that transitions from deep insertion in anionic bilayers to superficial binding with increasing lysyl-PG; F5W Mag exerts strong dual disruption of acyl chains and interfacial groups, with maximal activity at intermediate lysyl-PG levels; and NA preferentially perturbs the interfacial region, maintaining high potency at elevated lysyl-PG concentrations.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/scipharm93040049/s1>, Figure S1: Peak positions of the ν_sCH_2 vibration bands of the methylene groups as a function of the temperature of the pure supported lipid bilayers of DMPG (■), CL (●) and lysyl-PG (▲) in buffer (10 mM HEPES, 500 mM NaCl, 1 mM EDTA, pH 7.4); Figure S2: Peak positions of the asymmetric phosphate stretching ($\nu_{as}PO_2^-$) bands of (DMPG:CL, 80:20) membranes in the absence (■), and presence of increasing concentrations (1%, 5%, 10%) of LL-37 (pink), F5W Mag (blue), and NA (green). Symbols correspond to peptide concentrations: 1% (●), 5% (▲), and 10% (★). All spectra were recorded in buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, and pH 7.4); Figure S3: Peak positions of the asymmetric phosphate stretching ($\nu_{as}PO_2^-$) bands of (DMPG:CL:Lysyl-PG, 72:18:10) membranes in the absence (■), and presence of increasing concentrations (1%, 5%, 10%) of LL-37 (pink), F5W Mag (blue), and NA (green). Symbols correspond to peptide concentrations: 1% (●), 5% (▲), and 10% (★). All spectra were recorded in buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, and pH 7.4); Figure S4: Peak positions of the asymmetric phosphate stretching ($\nu_{as}PO_2^-$) bands of (DMPG:CL:Lysyl-PG, 64:16:20) membranes in the absence (■), and presence of increasing concentrations (1%, 5%, 10%) of LL-37 (pink), F5W Mag (blue), and NA (green). Symbols correspond to peptide concentrations: 1% (●), 5% (▲), and 10% (★). All spectra were recorded in buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, and pH 7.4); Figure S5: Peak positions of the asymmetric phosphate stretching ($\nu_{as}PO_2^-$) bands of (DMPG:CL:Lysyl-PG, 56:14:30) membranes in the absence (■), and presence of increasing concentrations (1%, 5%, 10%) of LL-37 (pink), F5W Mag (blue), and NA (green). Symbols correspond to peptide concentrations: 1% (●), 5% (▲), and 10% (★). All spectra were recorded in buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, and pH 7.4).

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Abbreviations

The following abbreviations are used in this manuscript:

lysyl-PG	Lysyl-phosphatidylglycerol
AMP(s)	Antimicrobial peptide(s)
F5WMag	F5W Magainin
NA	NA-CATH:ATRA-1-ATRA-1
FTIR	Fourier-transform infrared spectroscopy
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
CL	Cardiolipin
STX	Staphyloxanthin
PG	Phosphatidylglycerol
DMPG	1,2-dimyristoyl-sn-glycero-3-phosphoglycerol
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MprF	Multiple Peptide Resistance Factor
3adLPG	Synthetic analog of lysyl-phosphatidylglycerol
$\nu_s\text{CH}_2$	Symmetric stretching vibration of methylene groups
$\nu\text{C=O}$	Carbonyl stretching vibration
$\nu_{\text{as}}\text{PO}_2^-$	Asymmetric stretching vibration of phosphate groups
T_m	Main transition temperature
$\text{L}\beta\text{--L}\alpha$	Gel phase ($\text{L}\beta$)–Liquid crystalline phase ($\text{L}\alpha$)
NMR	Nuclear magnetic resonance
EC_{50}	Half-maximal effective concentration
CD	Circular dichroism

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